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For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin–proteasome connection

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The surveillance of the structural fidelity of the proteome is of utmost importance to all cells. The endoplasmic reticulum (ER) is the organelle responsible for proper folding and delivery of proteins to the secretory pathway. It contains a sophisticated protein proofreading and elimination mechanism. Failure of this machinery leads to disease and, finally, to cell death. Elimination of misfolded proteins requires retrograde transport across the ER membrane and depends on the central cytoplasmic proteolytic machinery involved in cellular regulation: the ubiquitin–proteasome system. The basics of this process as well as recent advances in the field are reviewed.
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Introduction

It is of great importance for the cell to regulate the individual entities of its proteome as well as to control the structural fidelity of each of its members. Proteins destined for secretion, the plasma membrane or the cell surface are translocated from the cytoplasm into the endoplasmic reticulum (ER), the central organelle for further delivery of these proteins to their site of action. Since proteins are translocated into the ER in an unfolded state, it is the primary function of this organelle to modify and fold the translocated proteins to acquire their biologically active conformation (Haigh and Johnson, 2002). In the ER, proteins undergo a quality control procedure that discriminates between properly folded proteins and terminally misfolded species as well as unassembled protein subunits (Ellgaard *et al*., 1999). The misfolded polypeptides and orphan subunits are subsequently subjected to ER-associated degradation (ERAD). The ERAD process requires retrotranslocation of the malfolded proteins across the ER membrane into the cytoplasm and subsequent degradation by the 26S proteasome (Sommer and Wolf, 1997; Brodsky and McCracken, 1999; Plemper and Wolf, 1999). ER degradation contributes to the molecular pathogenesis of many loss- and gain-of-toxic-function disorders (Aridor and Hannan, 2000; Kostova and Wolf, 2002; Rutishauser and Spiess, 2002). A detailed knowledge of this process is thus of great importance not only for our understanding of this basic cellular mechanism but also for the development of new strategies to treat a diverse set of diseases.

Protein folding and the unfolded protein response

Many proteins synthesized in the cytoplasm are transported via the secretory pathway to other compartments of the cell, the plasma membrane, or the extracellular space. Secretory proteins are first translocated into the ER in an unfolded state via an aqueous channel, the Sec61 translocon. In the ER lumen, proteins are properly folded, assembled into multisubunit complexes and covalently modified by a large array of ER-resident chaperones and enzymes, before they can enter the secretory pathway (Glick, 2002; Haigh and Johnson, 2002). The major components of this process in the ER are signal peptidase, which cleaves off the signal peptide from the newly translocated proteins; the oligosaccaryl-transferase complex (OST) which carries out *N*-glycosylation; and protein disulfide isomerase (PDI), which participates in disulfide bond formation. The two most studied examples of chaperones that assist proteins in their folding are the Hsp70 chaperone BiP (Kar2p in yeast), which recognizes hydrophobic patches on proteins, and calnexin, which binds carbohydrate moieties. Proteins are allowed to exit the ER and enter the secretory pathway only when they are properly folded and modified (Ellgaard *et al*., 1999). The importance of proper folding before ER exit is demonstrated by the existence of an unfolded protein response (UPR), a system that controls the level of the auxiliary folding proteins (Sidrauski *et al*., 2002). In *Saccharomyces cerevisiae*, the concentration of unfolded proteins in the ER is sensed by Ire1p, a transmembrane kinase localized to the ER/nuclear envelope, by virtue of a competition between the Ire1p lumenal domain and unfolded proteins for binding to Kar2p (BiP). Depletion of free Kar2p (BiP) molecules due to sequestration by increasing amounts of unfolded proteins leads to the dimerization of Ire1p, a conformational change that transmits a signal across the membrane and activates the cytoplasmic kinase activity. The kinase induces a non-canonical splicing of the *HAC1* mRNA, allowing synthesis of the Hac1p transcription factor, which upregulates genes containing a UPR response element. This cascade of events leads to an increase in the levels of proteins required for folding and quality control (Travers *et al*., 2000; Sidrauski *et al*., 2002). The UPR and ER degradation are tightly interconnected. Overexpression or accumulation of unfolded proteins due to the absence of a component of the ER degradation machinery induces the UPR (Knop *et al*., 1996a) which, in turn, upregulates the overall level of components of the ER degradation machinery (Friedländer *et al*., 2000; Travers *et al*., 2000). Under normal conditions, cells are able to cope with the amount of naturally present unfolded proteins and do not require induction of the UPR. However, when the level of misfolded proteins
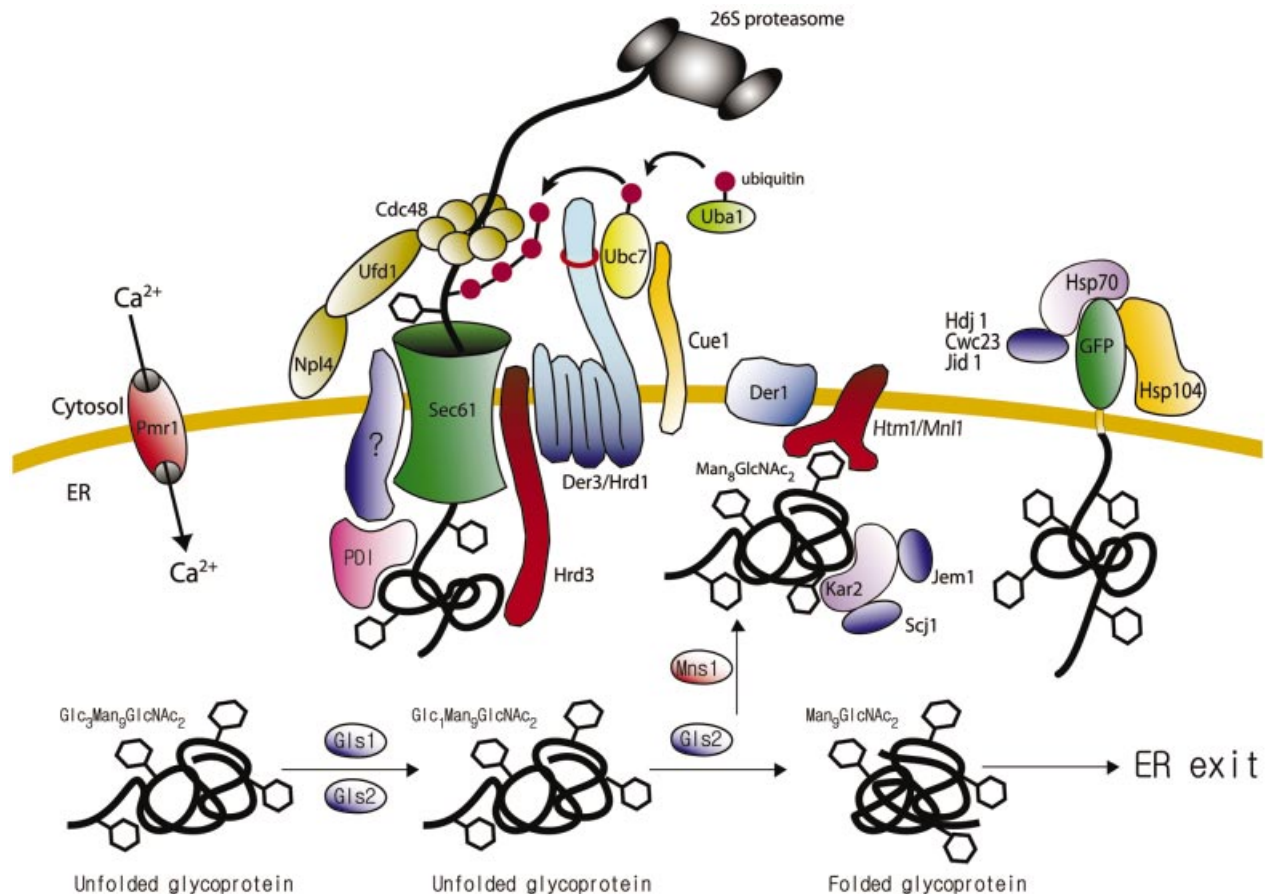
Fig. 1. Components of ER quality control and degradation in yeast.

rises above a certain level, the UPR becomes essential. In yeast, loss of function of components of both systems is lethal (Friedländer *et al*., 2000; Travers *et al*., 2000).

ER quality control

Prion diseases like Scrapie (sheep), bovine spongiform encephalopathy (BSE, cattle), or Creutzfeldt–Jakob disease (CJD, human), and other neurodegenerative diseases such as Parkinson and Alzheimer, are the result of precipitated protein aggregates. On the other hand, human diseases such as cystic fibrosis and lung emphysema are caused by the rapid disappearance of crucial proteins, like the cystic fibrosis transmembrane conductance regulator (CFTR) and α-1-antitrypsin, respectively (Jensen *et al*., 1995; Ward *et al*., 1995; Qu *et al*., 1996; Kostova and Wolf, 2002; Rutishauser and Spiess, 2002). The discovery of a degradation process for mutated and misfolded ER proteins in yeast (Sommer and Wolf, 1997; Brodsky and McCracken, 1999; Plemper and Wolf, 1999; Kostova and Wolf, 2002) has shed light on the molecular mechanism underlying such seemingly different diseases.

Protein function depends greatly on a precise three-dimensional conformation, achieved following an uninterrupted, competent folding and assembly process. Mistakes are, however, a fact of life. Mutations leading to an incorrect final structure result in inactive proteins and, if not properly dealt with, in protein aggregates. To minimize folding mistakes, a complex chaperone system has evolved to assist the folding process and to prevent dead-end conformations (Kopito, 2000). This folding machinery is especially active in the ER, into which nearly all proteins enter in an unfolded state. Protein stretches that leave the Sec61 translocon are immediately occupied by chaperones, preventing hydrophobic surfaces from creating erroneous intra- and/or intermolecular contacts. However, even chaperone-assisted folding cannot guarantee perfection, especially when faced with a mutant protein. To overcome this problem, a quality control system has evolved in the ER to function as a checkpoint that detects improperly folded proteins and targets them for elimination (Ellgaard *et al*., 1999). The most influential ER folding components are the Hsp70 chaperone BiP (Kar2p), the lectin like chaperones calnexin and calreticulin, and enzymes involved in disulfide bond formation, such as PDI and oxidoreductase Erp57. All of these components also participate in ER quality control. Quite unexpectedly, the elimination of misfolded proteins from the ER is dependent on the cytoplasmic ubiquitin–proteasome system and, therefore, requires protein retrotranslocation (dislocation) across the ER membrane (Hiller *et al*., 1996; Werner *et al*., 1996; Wiertz *et al*., 1996; Plemper *et al*., 1997).

Our current knowledge about the discovery of misfolded or unassembled proteins and their retention in the ER is still very limited. However, it is clear that the control mechanism works by structural rather than functional

criteria. Mutations in CFTR and α-1-antitrypsin, for example, which do not perturb the biological activity of the proteins *per se*, lead to ER retention and elimination of the mutant molecules, thus causing disease (Jensen *et al.*, 1995; Ward *et al.*, 1995; Qu *et al.*, 1996). To date, a chaperone-mediated retention mechanism has been described only for mutant glycoproteins in mammalian systems. As proteins become translocated across the ER membrane, core oligosaccharides of the Glc_3Man_9-$GlcNAc_2$ structure are co-translationally attached to the side chains of asparagine residues within the Asn-X-Ser/Thr consensus sequence. As folding progresses, two of the outermost glucose residues of the N-linked glycan are trimmed by the glucosidases I and II. In mammalian cells, exposure of the innermost glucose leads to the binding of the monoglucosylated protein to calnexin and calreticulin. If the glycoproteins contain cysteine residues, mixed disulfide bonds are transiently formed by the thiol oxidoreductase ERp57, a member of the PDI family which specifically interacts with calnexin, calreticulin and monoglucosylated glycoproteins, and functions as a disulfide isomerase. When the remaining glucose residue is trimmed by glucosidase II, the complex dissociates, releasing a protein with the carbohydrate structure $Man_9GlcNAc_2$. If the glycoprotein is not properly folded, the *N*-glycan is reglucosylated at the same position by a UDP-glucose glucosyltransferase (UGGT). This induces a new round of calnexin/calreticulin binding, which prevents the escape of the glycoprotein from the ER. If this cycle persists due to the inability of the glycoprotein to reach its final native conformation, mannosidase I, a slow-acting enzyme, cleaves the α1,2-linked mannose of the middle branch, generating a glycan with the structure $Man_8GlcNAc_2$. Direct recognition of $Man_8GlcNAc_2$ by a specific lectin or attenuated release of the reglucosylated form ($Glc_1Man_8GlcNAc_2$) from calnexin are events which determine the delivery of malfolded glycoproteins to the elimination machinery (Cabral *et al.*, 2001).

In recent years, the yeast *S.cerevisiae* has set the pace in the field of protein quality control in the ER (Figure 1). One of the model proteins used to study ER quality control in this organism is a mutated vacuolar enzyme, carboxypeptidase yscY-S255R, commonly known as CPY*. This misfolded protein is translocated into the ER lumen normally; it is fully glycosylated but is never transported to the vacuole. Instead, it is retained in the ER and degraded by the proteasome (Hiller *et al.*, 1996; Knop *et al.*, 1996a). It is interesting that ER quality control leads to degradation of misfolded proteins by the cytoplasmic proteasome machinery rather than by the vacuole (lysosome). A carbohydrate-based retention mechanism, which appears very similar to the mechanism described for mammalian cells, has also been identified for CPY*. Glucosidases I and II (Gls1, Gls2), and α-1,2 mannosidase (Mns1) are necessary for the disposal of CPY* (Knop *et al.*, 1996b; Jakob *et al.*, 1998). As opposed to the mammalian system, there is no known glucosyltransferase in yeast that could reglucosylate the carbohydrate chains. Thus, trimming by glucosidases I and II, and later by α-1,2 mannosidase, is currently believed to be the timer for folding or, if unsuccessful, for degradation. The recently discovered lectin-like yeast protein Htm1/Mnl1p, and its mouse homolog EDEM, is believed to bind $Man_8GlcNAc_2$

and provides us with a link between recognition and targeting for degradation (Hosokawa *et al.*, 2001; Jakob *et al.*, 2001; Nakatsukasa *et al.*, 2001) (Figure 1). Htm1p/Mnl1p is also necessary for the efficient degradation of other misfolded glycoproteins with differing topologies, such as Pdr5*, a mutated form of the ATP-binding cassette transporter Pdr5 (Plemper *et al.*, 1998), and Stt3-7p, a mutant subunit of the OST complex (Jakob *et al.*, 2001). On the other hand, degradation of Sec61-2p (Biederer *et al.*, 1996), which is not *N*-glycosylated, is totally independent of Htm1p/Mnl1p, a finding that emphasizes the necessity of this lectin specifically for the recognition of misfolded glycoproteins (Jakob *et al.*, 2001). At present, the overall impact of this carbohydrate-based recognition on the quality control and degradation process as a whole is not clear. Likewise, our knowledge of the mechanism underlying the discovery of misfolded or unassembled non-glycoproteins is minimal. An obvious possibility is recognition of exposed hydrophobic patches by chaperone-like molecules. The major Hsp70 chaperone of the ER, BiP (Kar2p), is known to play a vital role in binding hydrophobic protein stretches to allow post-translational protein import into the ER and for protein folding and assembly within the ER (Johnson and van Waes, 1999; Haigh and Johnson, 2002). Its general function in protein quality control, therefore, seemed pretty straightforward. It is required for the degradation of soluble proteins such as mutant pro-α-factor and CPY* in yeast (Plemper *et al.*, 1997; Brodsky *et al.*, 1999). Surprisingly, Kar2p becomes dispensable when CPY* is anchored to the ER membrane. Basically, conversion of the same misfolded protein, CPY*, from a soluble form to a membrane-bound form abolishes the requirement for Kar2p (BiP) in ER degradation (C.Taxis and D.H.Wolf, manuscript submitted for publication). Degradation of other membrane proteins is also independent of Kar2p (Plemper *et al.*, 1998). This indicates that Kar2p (BiP) and its DnaJ-like partners, Jem1p and Scj1p (Nishikawa *et al.*, 2001), do not have a general function in the quality control process. Instead, Kar2p activity seems limited to soluble proteins (Figure 1). One function of Kar2p appears to reside in its ability to remain bound to misfolded proteins after their unsuccessful folding trials, essentially keeping them in soluble form. Scj1p and Jem1p may be necessary for triggering the release of Kar2p from such substrates in order for dislocation and degradation to take place. BiP and its partners may also play a role in the delivery of the soluble substrates to other, as yet unknown, components linking recognition to elimination (Nishikawa *et al.*, 2001). BiP has also been implicated in acting as a seal for the protein translocation channel from the lumenal side (Johnson and van Waes, 1999; Haigh and Johnson, 2002). If BiP (Kar2p) has some function as a gatekeeper for the retrograde transport of malfolded proteins out of the ER, then this role would be limited to soluble proteins (C.Taxis and D.H.Wolf, manuscript submitted for publication). The requirement for Ca^{2+} ions in the ER for degradation of soluble CPY* may be partly explained by their regulatory role in Kar2p activity (Dürr *et al.*, 1998). PDI, an oxidoreductase involved in disulfide bond formation in the ER (Regeimbal and Bardwell, 2002), is emerging as another component with multiple functions. While elimination of CPY* (Gillece *et al.*, 1999) and unassembled

Ig-μ chains (Fagioli *et al.*, 2001) requires the enzymatic activity of PDI, elimination of the non-glycosylated and cysteine-free mutant pro-α-factor requires binding of PDI without enzymatic activity, suggesting a chaperone-like role for PDI under these circumstances (Gillece *et al.*, 1999) (Figure 1). PDI has also been described to function as a redox-driven chaperone in the unfolding of the A1 chain of cholera toxin in the ER lumen, prior to its transport to the cytoplasm. In this scenario, PDI binds and unfolds the substrate in its reduced state. The complex is then targeted to the ER membrane, where it binds to a protein at the lumenal side of the membrane. Oxidation of PDI by Ero1p releases the substrate, possibly directly into the retrotranslocon (Tsai and Rapoport, 2002). However, this redox-dependent mechanism has been questioned by Lumb and Bulleid's (2002) finding that PDI binding and release to two other substrates is not driven by the redox state of the protein. The necessity for Eps1p, a membrane-bound protein from the PDI family, in the elimination of a mutant form of the yeast membrane ATPase Pma1p (Wang and Chang, 1999), underlines the general necessity of this class of proteins as part of the ER protein quality control machinery. Calnexin represents yet another example of a multifunctional chaperone. In mammalian cells, it binds glycan moieties of proteins as expected from a lectin. In yeast, it is dispensable for CPY* degradation but it is involved in the elimination of the non-glycosylated mutant pro-α-factor (McCracken and Brodsky, 1996), suggesting additional non-lectin activity for this protein.

The protein quality control machinery of the ER is also involved in the regulated degradation of ER-resident enzymes. The best-studied example in yeast is a key enzyme in the sterol biosynthetic pathway, HMGCoA-reductase 2. Signals from the mevalonate pathway are presumed to lead to conformational changes which channel the protein into the ER quality control pathway (Hampton, 2002). Elimination of this enzyme requires all of the major components of the ER degradation machinery (Hampton *et al.*, 1996; Hampton and Bhakta, 1997; Gardner *et al.*, 2000; Bays *et al.*, 2001a,b).

Retrotranslocation

It was initially believed that misfolded and orphan proteins of the ER were degraded by ER-resident proteinases and peptidases (Bonifacino and Klausner, 1994). However, the presence of unspecific proteinases in the ER was hard to reconcile with its primary function in folding and assembly. Rather, delivery of misfolded proteins to the lysosome/vacuole, the compartment of the cell associated with degradation, seemed more likely. The discovery of the involvement of the cytoplasmic ubiquitin–proteasome system in the degradation of mutant ER membrane proteins was an unexpected surprise (Sommer and Jentsch, 1993; Jensen *et al.*, 1995; Ward *et al.*, 1995). The findings, in *S.cerevisiae*, that the misfolded vacuolar peptidase CPY* (Hiller *et al.*, 1996) and the mutant secretory protein pro-α-factor (Werner *et al.*, 1996) were retained in the ER lumen and degraded in the cytoplasm by the ubiquitin–proteasome system, were crucial in establishing the concept of retrograde transport out of the ER. Genetic studies in yeast revealed a significantly retarded

degradation of CPY* (Plemper *et al.*, 1997), mutated pro-α-factor (Pilon *et al.*, 1997) and a mutant polytopic membrane protein, Pdr5* (Plemper *et al.*, 1998), in mutants defective in the Sec61 translocon. Co-immuno-precipitation studies indicated that Sec61β was associated with the major histocompatibility complex class I (MHC class I) heavy chains, with wild-type and mutant forms of the CFTR and with a mutant form of ribophorin I (RI332) during their dislocation from the ER (Kostova and Wolf, 2002). These studies support the notion that the Sec61 import channel may also be the export channel. It is very likely, though, that the retrotranslocation channel differs in its composition from the import channel. The Sec61 translocon is composed of three different subunits: Sec61α, Sec61β and Sec61γ (Sec61p, Sbh1p and Sss1p in yeast). Studies on the retrotranslocon have identified a genetic interaction between Hrd3p and Sec61p (Plemper *et al.*, 1999a; P.Deak and D.H.Wolf, unpublished data) (Figure 1). The presence of two subsets of translocons can easily explain how this complicated two-way traffic across the membrane is regulated. Since CPY* leaves the translocon completely after import into the ER lumen (Plemper *et al.*, 1999b), retrotranslocation also necessi-tates retargeting of the protein to the Sec61 channel. Possible components of this retargeting assembly are Der1p, an ER membrane protein of unknown function (Knop *et al.*, 1996a), Kar2p (BiP), PDI, the lectin Htm1p/Mnl1p, and Hrd3p, an ER membrane protein that functions together with the ubiquitin–protein ligase Der3p/Hrd1p (Plemper *et al.*, 1999a; Gardner *et al.*, 2000; Deak and Wolf, 2001). Given the cooperativity between targeting, dislocation and degradation, it is very likely that these components do not act independently of each other. A recent report suggests that a fusion protein with an enhanced GFP and an MHC class I heavy chain (EGFP-HCI) domain may be retrotranslocated without unfolding of the EGFP domain (Fiebiger *et al.*, 2002). The inner diameter of the translocon pore is estimated to fluctuate between 15 Å, in its inactive state, and 40–60 Å during protein translocation (Johnson and van Waes, 1999; Haigh and Johnson, 2002). Therefore, it is possible that in its active state the translocon could accommodate a folded GFP molecule with a length of 24 Å and a diameter of 42 Å. The retrotranslocon could even accommodate larger molecules through rearrangements of translocon subunits. However, additional biochemical proof is essential for the characterization of the dislocation channel.

Ubiquitylation and targeting to the proteasome

Following retrotranslocation from the ER, nearly all misfolded proteins are polyubiquitylated prior to degrad-ation (Thrower *et al.*, 2000; Kostova and Wolf, 2002). One exception is the mutated pro-α-factor (Werner *et al.*, 1996), which, although not ubiquitylated, is still targeted to the proteasome. Genetic and biochemical studies in yeast characterized the ubiquitylation machinery and localized it to the cytoplasmic side of the ER membrane. Ubc6p, a tail-anchored E2 (Sommer and Jentsch, 1993) and Ubc7p, a soluble cytoplasmic E2 recruited to the ER membrane via Cue1p (Biederer *et al.*, 1997), are the two best-studied components of the ubiquitylation machinery

(Biederer *et al*., 1996; Hiller *et al*., 1996; Plemper *et al*., 1998). Recently, Ubc1p has also been implicated as an E2 involved in CPY* ubiquitylation (Friedländer *et al*., 2000). The ubiquitin–protein ligase (E3) cooperating with Ubc1p and Ubc7p is the RING-H2 finger domain ER membrane protein Der3/Hrd1p (Bays *et al*., 2001a; Deak and Wolf, 2001). Der3/Hrd1p activity is dependent on the presence of an interacting ER membrane protein, Hrd3p (Hampton *et al*., 1996). Hrd3p is believed to play a role in signaling events between the ER lumen and the cytoplasmic ubiquitylation machinery (Plemper *et al*., 1999a; Gardner *et al*., 2000; Deak and Wolf, 2001) and is necessary for the degradation of both soluble and polytopic membrane proteins (Figure 1). Doa10/Ssm4p is the second E3 described in yeast as involved in ER-associated degradation. In addition to its involvement in the ubiquitylation of the Deg1 domain of the transcriptional repressor MATα2, Doa10/Ssm4p is also involved in the degradation of the tail-anchored ER membrane protein Ubc6p (Swanson *et al*., 2001). Interestingly, neither the ubiquitin–protein ligase Der3/Hrd1p, its interaction partner Hrd3p, nor the Sec61 channel participate in the elimination of Ubc6p (Walter *et al*., 2001). The elimination of the vacuolar membrane ATPase subunit Vph1p (Hill and Cooper, 2000; Wilhovsky *et al*., 2000) is also independent of the function of the Der3/Hrd1p–Hrd3p complex. It is still an open question whether Doa10/Ssm4p is involved. The ubiquitin conjugating enzymes MmUbc6 and MmUbc7 (Tiwari and Weissman, 2001), and the RING-finger E3, gp78 (Fang *et al*., 2001), are the mammalian orthologs of the yeast ER-ubiquitylation machinery. Interestingly, gp78 recruits MmUbc7 to the ER membrane through a region that exhibits homology to yeast Cue1p, representing a case of convergent evolution of two functions into a single polypeptide. These proteins have been shown to participate in proteasomal degradation of mammalian ERAD substrates (Fang *et al*., 2001).

ER degradation requires directionality from the ER lumen or membrane to the proteasome. Once the protein is threaded into the retrotranslocon, there needs to be a pulling or pushing force to direct the substrate to the proteasome. It is clear now that lack of polyubiquitylation not only prevents proteasomal degradation (Hiller *et al*., 1996; Biederer *et al*., 1997) but also leads to failure in transport out to the cytoplasm. The substrate seems to remain or slips back into the ER (Biederer *et al*., 1997; Bordallo *et al*., 1998; Shamu *et al*., 2001), which suggests that polyubiquitylation is necessary for retrotranslocation. Modification of the protein may occur when the N-terminus or the first lysine residue becomes accessible to the ubiquitylation machinery (Hershko and Ciechanover, 1998). Progressive polyubiquitylation may serve as a ratcheting mechanism moving the polypeptide from the retrotranslocation channel into the cytoplasm, where the long and bulky polyubiquitin chains prevent the polypeptide from slipping back into the ER. Consistent with this idea are the findings that a hypo-ubiquitylated CPY* fails to be completely transported into the cytosol (Jarosch *et al*., 2002). Recent data indicate that Cdc48 (p97), a member of the AAA family of ATPases, together with two partner proteins, Ufd1p and Npl4p, forms a complex essential for the extraction and degradation of a number of substrate proteins (Bays *et al*., 2001a; Ye *et al*.,

2001; Braun *et al*., 2002; Jarosch *et al*., 2002; Rabinovich *et al*., 2002). In the absence of the Cdc48 complex, polyubiquitylated substrates accumulate at the ER membrane, suggesting a role downstream of polyubiquitylation. At the moment there is controversy concerning the temporal organization and association of this complex with the ER membrane and the subunit responsible for ubiquitin binding (Dai and Li, 2001; Meyer *et al*., 2002). Cdc48 exists as homo-hexameric rings, which undergo strong conformational changes upon ATP hydrolysis (Rouiller *et al*., 2000; Zhang *et al*., 2000), critical for substrate dislocation from the ER (Ye *et al*., 2001). There is convincing evidence that the Cdc48–Ufd1–Npl4 complex can separate a tightly associated protein at the ER membrane, a processed dimer of the Spt23 transcription factor, and, by this, release active transcription factors into the cytosol (Rape *et al*., 2001). The general picture that emerges is the following: after polyubiquitylation and partial dislocation of the substrate from the retrotranslocon, the ER-associated Cdc48–Ufd1p–Npl4 complex binds the ubiquitylated protein. Rounds of ATP hydrolysis then induce conformational changes in the Cdc48 complex, which progressively pulls the polyubiquitylated substrate away from the ER membrane and hands it over to the proteasome for degradation (Figure 1). In mutants defective either in proteasome activity or in one of the 19S cap ATPases, CPY* accumulates to a large extent in the cytoplasm (Jarosch *et al*., 2002). Similarly, MHC class I molecules are found as soluble ubiquitylated intermediates in HCMV US11 expressing cells when the proteasome is inhibited (Wiertz *et al*., 1996; Shamu *et al*., 2001). These data confirm that the proteasome acts after release of the ubiquitylated substrate from the ER membrane. In a few cases, however, the proteasome itself seems to have an active role in the dislocation of membrane-bound proteins (Mayer *et al*., 1998; Walter *et al*., 2001). Since the role of Cdc48 in the extraction of these membrane proteins has yet to be tested, it is not clear whether there are different modes for ER-membrane extraction. It is certainly possible that other as yet unknown components exist as mediators between the Cdc48 complex and the proteasome.

Glycosylated proteins of the ER are retrotranslocated into the cytoplasm in a glycosylated state (Hiller *et al*., 1996; Wiertz *et al*., 1996). The discovery of de-*N*-glycosylated intermediates in the cytosol upon inhibition of the proteasome alludes to the activity of a cytoplasmic *N*-glycanase prior to proteasomal degradation. Recently, a highly conserved cytoplasmic N-glycanase (Png1p) has been identified in yeast. *PNG1* is not an essential gene but its product is required for efficient degradation of CPY* (Suzuki *et al*., 2000). Rad23, a protein that interacts with the proteasome via its ubiquitin-like (Ubl) domain escorts Png1p to the proteasome where it is thought to act in a complex to efficiently deglycosylate the substrate prior to degradation (Suzuki *et al*., 2001).

The involvement of cytoplasmic chaperones in the degradation of misfolded soluble ER lumenal proteins is an open question. In contrast, misfolded ER membrane proteins with tightly folded cytoplasmic domains seem to require additional chaperone activity for efficient degradation. Elimination of the CFTR expressed in yeast is
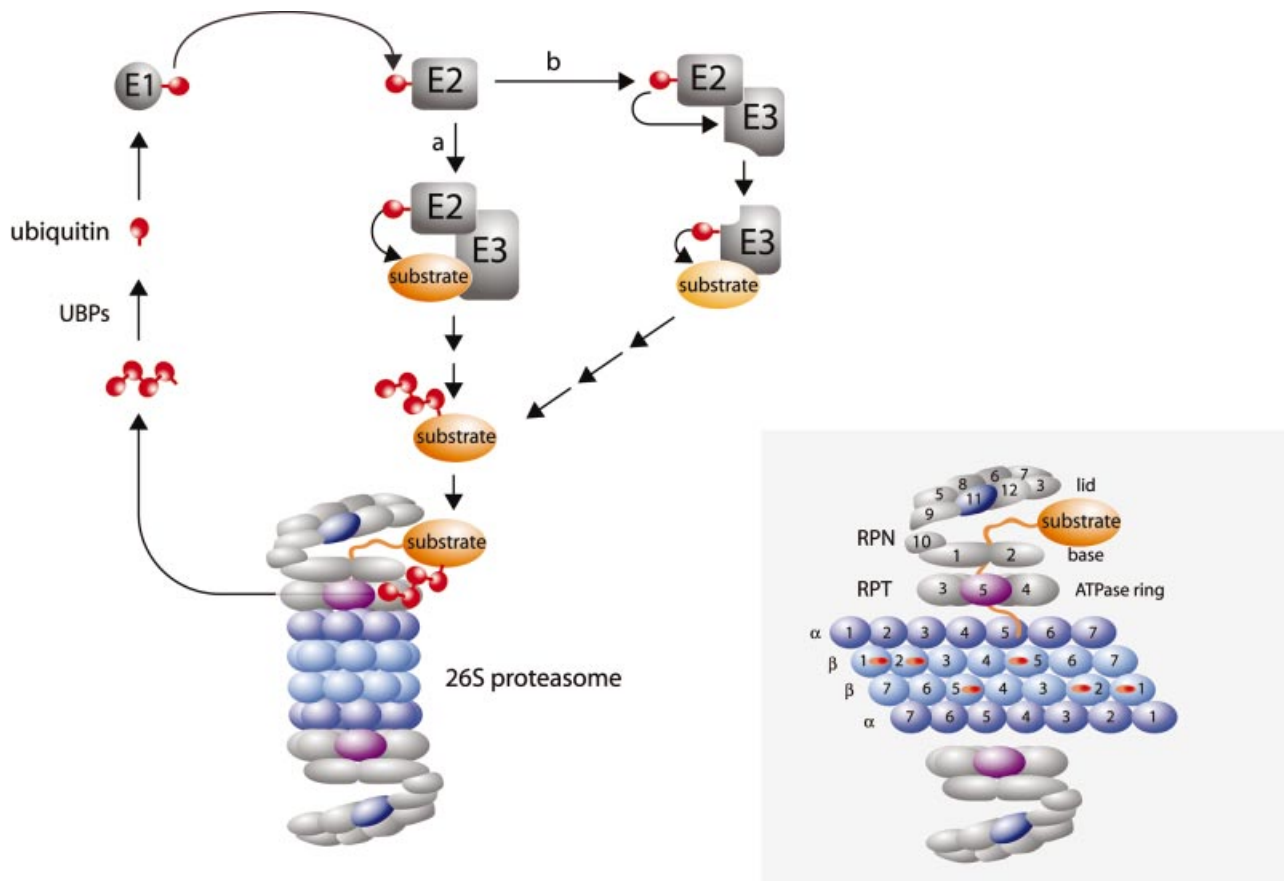
Fig. 2. Ubiquitylation machinery and the 26S proteasome.

dependent on the presence of cytoplasmic Hsp70 chaperones (Zhang *et al*., 2001). The use of modular substrates containing the misfolded CPY* domain fused to a membrane anchor, with (CTG*), or without (CT*), an added cytosolic GFP domain, has given clues about the possible requirements for cytosolic chaperones. While soluble CPY* and membrane-anchored CT* do not require additional cytoplasmic components other than the Cdc48 complex, degradation of membrane-bound CTG* is dependent on Hsp70, Hsp40, and, to a certain extent, Hsp104 chaperone activity (Figure 1) (C.Taxis and D.H.Wolf, manuscript submitted for publication). One may, therefore, speculate that the tighter the folding of the cytoplasmic domain of a malfolded ER membrane protein, the stronger is the requirement for cytoplasmic chaperone activity for degradation.

It has recently been shown that efficient ER degradation of soluble malfolded proteins requires an efficient ER–Golgi traffic (Caldwell *et al*., 2001; Vashist *et al*., 2001; Taxis *et al*., 2002). Possible explanations include the necessity of transport of the malfolded proteins from ER to Golgi for further modification, or the ER–Golgi cycling of an as yet unknown factor as a prerequisite for efficient degradation (Caldwell *et al*., 2001; Vashist *et al*., 2001). A recent study reports that overexpression of CPY* and other malfolded proteins leads to dependence on the activity of the Golgi-associated E3 Rsp5, rather than the Der3/Hrd1p–Hrd3p complex (Haynes *et al*., 2002). This finding is controversial because other reports show that degradation of overexpressed CPY* is still dependent on

Der3/Hrd1p (Friedländer *et al*., 2000). New data from Taxis *et al*. (2002) lead to yet another interpretation: they propose that efficient ER degradation simply requires an intact ER with all its components, which is, in turn, dependent on the existence of a normal ER–Golgi traffic. Future experiments are clearly necessary to solve this somewhat controversial issue.

Degradation by the 26S proteasome

Proteins selected for degradation are tagged with ubiquitin. This 76 amino acid polypeptide is bound in an isopeptide linkage through its C-terminal glycine to the N-terminus or to the ε-amino group of internal lysine residues of target proteins. First, ubiquitin is activated by an ATP-dependent *trans*-esterification reaction and attached to a cysteine residue of a ubiquitin-activating enzyme (E1, Uba). Next, ubiquitin is transferred to a cysteine of a ubiquitin-conjugating enzyme (E2, Ubc) and finally, to the substrate directly or via the intervention of a third class of enzymes, ubiquitin–protein ligases (E3, Ubl). Proteasomal degradation requires polyubiquitylation of the substrate, accomplished by attaching additional ubiquitin moieties usually to Lys48 of the preceding ubiquitin (Pickart, 2000; Thrower *et al*., 2000). The 26S proteasome binds the polyubiquitylated substrate, unfolds and degrades it (Figure 2) (Heinemeyer *et al*. 1991; Baumeister *et al*., 1998). This is a complex proteolytic assembly that consists of two parts, the 20S core and the 19S regulatory particle. The 20S core is a cylinder

composed of four stacked rings, each containing seven different α or β subunits, with an overall α7β7β7α7 geometry. The three different active sites are located inside the cylindrical core within the β-subunit rings (Figure 2) (Groll *et al.*, 1997; Baumeister *et al.*, 1998). N-terminal stretches of the external α-subunits regulate the entry of substrates into the proteolytic core (Groll *et al.*, 2000). The 19S cap is involved in the recognition, binding and unfolding of ubiquitylated proteins, and in the regulation of the opening of the 20S core. It is composed of 17 different subunits, functionally divided into two parts: the base and the lid. The base consists of a ring of six ATPases (Rpt1–Rpt6), which dock onto the α-rings of the 20S core, and three non-ATPase subunits (Rpn1, Rpn2 and Rpn10) (Figure 2). The specific functions of the ATPase subunits in binding and unfolding are slowly emerging (Braun *et al.*, 1999). Rpt5 binds the ubiquitylated substrate (Lam *et al.*, 2002). Rpt2 is believed to control both substrate entry and product release from the 20S channel (Köhler *et al.*, 2001). Rpn1 interacts with Rad23 and Dsk2, two proteins harboring ubiquitin-like domains (Ubl) and capable of binding and delivering ubiquitylated cargo to the proteasome. Recent data suggest that Rpn10 contributes to the binding of ubiquitin chains as well (Elsässer *et al.*, 2002). The lid is composed of eight subunits: Rpn3, Rpn5 to Rpn9, Rpn11 and Rpn12 (Glickman *et al.*, 1998) (Figure 2). Rpn11 contains a highly conserved metallo-isopeptidase motif and this activity is necessary for de-ubiquitylation and proteasomal proteolysis of substrates. It is currently believed that Rpn11 de-ubiquitylates the substrate after it has been threaded into the 20S channel, thereby resulting in an irreversible commitment to proteolysis. Failure to de-ubiquitylate probably causes a sterical block to further insertion of the substrate into the proteolytic core (Verma *et al.*, 2002; Yao and Cohen, 2002). Following release from the substrate, the poly-ubiquitin chain is hydrolyzed into single ubiquitin moieties which can take part in a new round of protein degradation (Figure 2).

The undisturbed interplay between the folding and retrotranslocation machinery of the ER and the ubiquitin–proteasome system in the cytosol is essential for the maintenance of a healthy cellular life/metabolism both in unicellular and multicellular organisms. We now understand that there are many unique scenarios and substrate-specific details and variations about the way in which cells pursue ER quality control, and a unifying theme, if not for the protein components involved then at least for the sequence of events ending with proteasomal degradation, is emerging. However, there are still a lot of open questions and unresolved issues concerning ER-associated degradation as a whole, which will keep the scientific community busy for quite a long time.

Acknowledgements

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